

WO9115572

Publication Title:

LIVE VACCINES

Abstract:

Abstract of WO9115572

Attenuated microorganism for use in immunoprophylaxis in which the attenuation is brought about by the presence of a mutation in the DNA sequence of the microorganism which encodes, or which regulates the expression of DNA encoding a protein that is produced in response to environmental stress, the microorganism optionally being capable of expressing DNA encoding a heterologous antigen. Data supplied from the esp@cenet database - Worldwide ad6

Courtesy of <http://v3.espacenet.com>

This Patent PDF Generated by Patent Fetcher(TM), a service of Stroke of Color, Inc.

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 1/20, 1/21, A61K 39/112 // A61K 39/10, 39/102, 39/106 A61K 39/108	A1	(11) International Publication Number: WO 91/15572 (43) International Publication Date: 17 October 1991 (17.10.91)
(21) International Application Number: PCT/GB91/00484 (22) International Filing Date: 28 March 1991 (28.03.91) (30) Priority data: 9007194.5 30 March 1990 (30.03.90) GB (71) Applicant (for all designated States except US): THE WELL-COME FOUNDATION LIMITED [GB/GB]; Unicorn House, 160 Euston Road, London NW1 2BP (GB). (72) Inventors; and (75) Inventors/Applicants (for US only) : DOUGAN, Gordon [GB/GB]; CHARLES, Ian, George [GB/GB]; Langley Court, Beckenham, Kent BR3 3BS (GB). HOR-MAECHE, Carlos, Estenio [GB/GB]; JOHNSON, Kevin, Stuart [GB/GB]; Department of Pathology, University of Cambridge, Laboratories Block, Addenbrooks Hospital, Hills Road, Cambridge CB2 2QQ (GB). CHATFIELD, Steven, Neville [GB/GB]; Langley Court, Beckenham, Kent BR3 3BS (GB).		(74) Agent: STOTT, M., J.; The Wellcome Foundation Limited, Langley Court, Beckenham, Kent BR3 3BS (GB). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: LIVE VACCINES (57) Abstract Attenuated microorganism for use in immunoprophylaxis in which the attenuation is brought about by the presence of a mutation in the DNA sequence of the microorganism which encodes, or which regulates the expression of DNA encoding a protein that is produced in response to environmental stress, the microorganism optionally being capable of expressing DNA encoding a heterologous antigen.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

LIVE VACCINES

The present invention relates to attenuated microorganisms, to methods for their production, to their use in the immunoprophylaxis of an animal or a human, and to vaccines containing them.

The principle behind vaccination or immunoprophylaxis is to induce an immune response in an animal to a pathogenic organism by inoculation with an attenuated strain of the organism thus providing protection against subsequent challenge. In 1950 Bacon *et al* (Br.J.Exp.Path. 31, 714-724) demonstrated that certain auxotrophic mutants of *S.typhi* were attenuated in mice when compared to the parental strain. Certain of these auxotrophic mutants have been proposed as being suitable candidates for the basis of a whole cell vaccine. (See for example Hosieth and Stocker, *Nature*, 1981 241, 238-239, and European patent publication 322,237). In addition to mutations in an essential auxotrophic pathway, other loci have been identified where mutations result in attenuation of microorganisms. Examples of such loci include regulons that exert pleiotrophic effects, e.g., the *cva/crp* system (Roy Curtiss III *et al*, *Vaccine* 6, 155-160, 1988) and the *ompR envZ* system (Dorman *et al*, *Infect.Immun.* 57, 2136-2140, 1989) and the *phoP* system (Fields *et al*, *Science* 243, 1059-1062, 1989).

In many microorganisms, between one and two dozen proteins are produced in response to a range of different environmental stresses, such as high temperature, nutrient deprivation, toxic oxygen radicals and metabolic disruption. These represent part of the coordinated regulation of various different genes induced in response to the particular stress to which the microorganism is subjected. The family of major stress proteins (also known as heat shock proteins) is amongst the most highly conserved in nature. Substantial homology exists amongst members of this family isolated from *E.coli*, *Drosophila* spp. and man (for a recent review see Neidhardt, G.C. & Van Bogelen, R.A. (1987) *Escherichia coli* and *Salmonella typhimurium*:

Cellular and Molecular Biology. F.C. Neidhardt et al. eds. pp. 1334-1345. American Society for Microbiology, Washington DC). For example: Hsp90, Hsp70 and Hsp60 are heat shock proteins found in all prokaryotes and eukaryotes. Amino acid sequence comparison between Hsp90 from E.coli and that from man shows that approximately half the amino acid residues are identical. Other members of the stress protein family are GrpE, GroEL, DnaK, GroES, Lon and DnaJ.

The genes encoding the family of heat shock proteins are transcribed by RNA polymerase co-operating with the σ^{32} factor, the product of the rpoH gene (reviewed by Neidhardt, F.C. and van Bogelen, R.A, 1987. In Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology, Neidhardt, F.C. et al. eds. pp. 1334-1345, American Society for Microbiology, Washington, D.C.). Recently, Lipinska et al. (Nucleic Acids Res. 1988 21, 10053-10067) have described a heat shock protein in E.coli, referred to as HtrA, that appears to be σ^{32} -independent. Examination of the promoter region of the htrA gene shows DNA sequence homology with the P.3 promoter of the rpoH gene; a promoter known to be recognised by $\sigma^{E(24)}$ factor. This similarity suggests that the htrA promoter may also be recognised by the RNA polymerase- $\sigma^{E(24)}$ holoenzyme.

Phenotypically, in E.coli, a mutation in the htrA locus abolishes the ability of bacterium to survive at temperatures above 42°C (Lipinska et al., 1989, J.Bacteriol. 171, 1574-1584). The gene maps at 4 min on the E.coli chromosome and encodes a protein with a relative molecular mass (Mr) of 51,163. This protein precursor undergoes N-terminal processing involving the removal of a signal peptide sequence (Lipinska et al., 1988, Nucleic Acids Res. 21, 10053-10067), to yield the mature form of the polypeptide upon secretion through the inner membrane of the bacterium. Independently, the htrA gene has been identified as degP by Strauch, K.L. and Beckwith, J. 1988 (Proc.Natl.Acad.Sci. USA 85, 1576-1580) who were examining E.coli mutants with decreased protease activity, degP mutants were isolated by TnphoA mutagenesis (Manoil, C. & Beckwith, J. 1985,

Proc.Natl.Acad.Sci. USA 82, 8129-8133) and were recognised by the increased stability of a hybrid Tsr-phoA (Tsr-AP2) recombinant protein in a degP background (Strauch, K.L. and Beckwith, J. 1988. Proc.Natl.Acad.Sci. USA 85, 1576-1680). In E.coli the genes identified as degP and htrA appear to be identical and encode a protein that is a member of the 'stress-response' family.

The present invention provides an attenuated microorganism for use in immunoprophylaxis in which the attenuation is brought about by the presence of a mutation in the DNA of the microorganism which encodes, or which regulates the expression of DNA encoding, a protein that is produced in response to environmental stress, the microorganism optionally being capable of expressing DNA encoding a heterologous antigen.

The microorganisms for use with the present invention are preferably bacteria especially Gram-negative bacteria which invade and grow within eucaryotic cells and colonise the muscosal surface. Examples of these include members of the genera Salmonella, Bordetella, Vibrio, Haemophilus and Escherichia. In particular the following species can be mentioned : S.typhi - the cause of human typhoid; S.typhimurium - the cause of salmonellosis in several animal species; S.enteritidis - a-cause of food poisoning in humans; S.choleraesuis - the cause of salmonellosis in pigs; Bordetella pertussis - the cause of whooping cough; Haemophilus influenzae - a cause of meningitis; and Neisseria gonorrhoeae - the cause of gonorrhoea.

The mutation of the DNA is a non-reverting mutation, namely one which cannot be repaired in a single step. Genetic mutations of this sort include deletion, inversion, insertion or substitution mutations. Deletion mutations can be generated using transposons. These are DNA sequences comprising from between 750 to thousands of base pairs which can integrate into the host's chromosomal DNA. The continuity of the DNA sequence of interest is thus disrupted with the loss of gene function. Transposons can be deleted from the host chromosomal DNA;

most frequently excision is imprecise leading to a non-reverting mutation. Substitution or insertion mutations can arise by use of an inactivated DNA sequence carried on a vector which recombines with or crosses-over with the DNA sequence of interest in the host's chromosomal DNA with the consequent loss of gene function.

Examples of proteins that are produced in response to environmental stress include heat shock proteins (which are produced in response to a temperature increase above 42°C); nutrient deprivation proteins (which are produced in response to levels of essential nutrients such as phosphates or nitrogen which are below that which the microorganism requires to survive); toxic stress proteins (which are produced in response to toxic compounds such as dyes, acids or possibly plant exudates); or metabolic disruption proteins (which are produced in response to fluctuations in for example ion levels affecting the microorganisms ability to osmoregulate, or vitamin or co-factor levels such as to disrupt metabolism).

Preferably a heat shock protein is the one encoded by the htrA gene as set out in Fig. 1. (SEQ ID No: 1) (also characterised as degP). Other proteins are encoded by genes known to be involved in the stress response such as grpE, groEL, (moPA), dnaK, groES, lon and dnaJ. There are many other proteins encoded by genes which are known to be induced in response to environmental stress (Ronson et al., Cell 49, 579-581). Amongst these the following can be mentioned: the ntrB/ntrC system of E.coli, which is induced in response to nitrogen deprivation and positively regulates glnA and nifLA (Buck et al., Nature 320, 374-378, 1986; Hirschman et al., Proc.Natl.Acad.Sci. USA, 82, 7525, 1985; Nixon et al., Proc.Natl.Acad.Sci. USA 83, 7850-7854, 1986, Reitzer and Magasanik, Cell, 45, 785, 1986); the phoR/phoB system of E.coli which is induced in response to phosphate deprivation (Makino et al., J.Mol.Biol. 192, 549-556, 1986b); the cpxA/sfrA system of E.coli which is induced in response to dyes and other toxic compounds (Albin et al., J.Biol.Chem. 261 4698, 1986; Drury et al., J.Biol.Chem. 260, 4236-4272, 1985). An analogous system in Rhizobium

is dctB/dctD, which is responsive to 4C-dicarboxylic acids (Ronson *et al.*, J.Bacteriol. 169, 2424 and Cell 49, 579-581, 1987). A virulence system of this type has been described in Agrobacterium. This is the virA/virG system, which is induced in response to plant exudates (le Roux *et al.*, EMBO J. 6, 849-856, 1987; Stachel and Zambryski., Am.J.Vet.Res. 45, 59-66, 1986; Winans *et al.*, Proc.Natl. Acad.Sci. USA, 83, 8278, 1986). Similarly the bvgC-bvgA system in Bordetella pertussis (previously known as vir) regulates the production of virulence determinants in response to fluctuations in Mg²⁺ and nicotinic acid levels (Arico *et al.*, 1989, Proc.Natl.Acad.Sci. USA 86, 6671-6675).

For use in the form of a live vaccine, it is clearly important that the attenuated microorganism of the present invention does not revert back to the virulent state. The probability of this happening with a mutation in a single DNA sequence is considered to be small. However, the risk of reversion occurring with a microorganism attenuated by the presence of mutations in each of two discrete DNA sequences, is considered to be insignificant. It is preferred therefore that the attenuation of the microorganism of the present invention is brought about by the presence of a mutation in the DNA sequence which encodes, or which regulates the expression of DNA encoding, a protein that is produced in response to environmental stress and by the presence of a mutation in a second DNA sequence. For bacteria, the second DNA sequence preferably encodes an enzyme involved in an essential auxotrophic pathway or is a sequence whose product controls the regulation of osmotically responsive genes, i.e. ompR, (Infect and Immun 1989 2136-2140). Most preferably, the mutation is in a DNA sequence involved in the aromatic amino acid biosynthetic pathway, more particularly the DNA sequences encoding aroA, aroC or aroD. (EP Publication Number 322237).

The attenuated microorganisms of the present invention are constructed by the introduction of a mutation into the DNA sequence by methods known to those skilled in the art (Maniatis, Molecular Cloning and

Laboratory Manual, 1982). Non-reverting mutations can be generated by introducing a hybrid transposon Tnp_{ho}A into, for example, S.typhimurium strains. Tnp_{ho}A can generate enzymatically active protein fusions of alkaline phosphatase to periplasmic or membrane proteins. The Tnp_{ho}A transposon carries a gene encoding kanamycin resistance. Transductants are selected that are kanamycin resistant by growing colonies on an appropriate selection medium.

Alternative methods include cloning the DNA sequence into a vector, eg. a plasmid or cosmid, inserting a selectable marker gene into the cloned DNA sequence, resulting in its inactivation. A plasmid carrying the inactivated DNA sequence and a different selectable marker can be introduced into the organism by known techniques (Maniatis, Molecular Cloning and Laboratory Manual, 1982). It is then possible by suitable selection to identify a mutant wherein the inactivated DNA sequence has recombined into the chromosome of the microorganism and the wild-type DNA sequence has been rendered non-functional in a process known as allelic exchange. In particular, the vector used is preferably unstable in the microorganism and will be spontaneously lost. The mutated DNA sequence on the plasmid and the wild-type DNA sequence may be exchanged by a genetic cross-over event. Additional methods eliminate the introduction of foreign DNA into vaccine strains at the site of mutations.

The invention therefore provides a process for the production of an attenuated microorganism according to the invention which comprises introduction of a mutation in the DNA sequence of the microorganism which encodes, or which regulates expression of a DNA sequence encoding, a protein that is produced in response to environmental stress, by either

- a) transposon mutagenesis; or

- b) transforming the microorganism with a vector incorporating a DNA sequence encoding, or regulating the expression of a DNA sequence encoding, a protein that is produced in response to environmental stress and which contains a non-reverting mutation; and screening to select the desired microorganisms.

The attenuated microorganism of the present invention is optionally capable of expressing a heterologous antigen. This expression is likely to be more favourable in htrA mutants because of the increased stability of recombinant antigens associated with the degP phenotype. Such antigens may be viral, bacterial, protozoal or of higher parasitic microorganisms. Such microorganisms may then form the basis of a bi- or multi-valent vaccine. Examples of useful antigens include E.coli heat labile toxin B subunit (LT-B), E.coli K88 antigens, FMDV (Foot and Mouth) peptides, Influenza viral proteins, P.69 protein from B.pertussis. Other antigens which could be usefully expressed would be those from Chlamydia, flukes, mycoplasma, roundworms, tapeworms, rabies virus and rotavirus.

A microorganism capable of expressing DNA encoding a heterologous antigen may be produced by transformation of the microorganism with an expression cassette. Expression cassettes will include DNA sequences, in addition to that coding for the heterologous antigen, which will encode transcriptional and translational initiation and termination sequences. The expression cassette may also include regulatory sequences. Such expression cassettes are well known in the art and it is well within the ability of the skilled man to construct them. The expression cassette may form part of a vector construct or a naturally occurring plasmid. An example of a genetically engineered attenuated Salmonella which is capable of expressing a heterologous antigen is described in EP publication 127,153. The expression cassette may also be engineered to allow the incorporation of the heterologous gene into the chromosome of the microorganism.

A further bivalent vaccine comprising an attenuated Salmonella typhi, capable of expressing the E.coli heat-labile enterotoxin subunit B is disclosed by Clements et al (Infection and Immunity, 46, No.2. 1984, 564-569). Ty21a, an attenuated S.typhi strain, has been used to express other antigens such as the Shigella sonnei form I antigen (Formal et al., Infection and Immunity, 34, 746-750, 1981).

According to a further aspect of the invention there is provided a vaccine which comprises an effective amount of an attenuated microorganism, preferably a bacterium, as herein described and a pharmaceutically acceptable carrier.

The vaccine is advantageously presented in a lyophilised form, for example in a capsular form, for oral administration to a patient. Such capsules may be provided with an enteric coating comprising for example Eudragate "S" Eudragate "L" Cellulose acetate, cellulose pthalate or hydroxy propylmethyl cellulose. These capsules may be used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, eg. as a suspension. Reconstitution is advantageously effected in a buffer at a suitable pH to ensure the viability of the organisms. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively, the vaccine may be prepared for parenteral administration, intranasal administration or intramammary.

The present invention also provides a method of prophylactic treatment of a host (particularly a human host) with an infection caused by a microorganism which comprises administering to said host an effective dose of a vaccine according to the invention. The dosage employed in such a method of treatment will be dependent on various clinical factors, including the size and weight of the host, the type of vaccine formulated. However, for attenuated S.typhi a dosage

comprising the administration of 10^9 to 10^{11} S.typhi organisms per dose is generally convenient for a 70kg adult human host.

The following examples provide experimental details in accordance with the present invention. It will be understood that these examples are not intended to limit the invention in any way.

Figure Legend

Figure 1. DNA sequence of the htrA gene and the amino acid sequence of the protein it encodes.

Figure 2. Sensitivity of S.typhimurium htrA mutant 046 to temperatures above 42°C and oxygen radicals

Figure 3. In vivo kinetics of S.typhimurium strains harbouring a mutation in htrA (BRD726) and htrA aro mutations (BRD807).

Example 1

Identification of the htrA gene in Salmonella typhimurium and generation of an htrA mutant.

TnphoA mutagenesis was used in the mouse virulent Salmonella typhimurium strain C5 (Miller et al., 1989, Infect.Immunol., 57, 2758-2763). Mutants were selected likely to harbour lesions in genes that have a signal peptide sequence, i.e. proteins likely to be targeted through a bacterial membrane. Isolation of the DNA flanking the TnphoA insertion identifies the gene that has been insertionally activated. This gene was isolated and its DNA sequence was determined by standard methods (see Figure 1. SEQ ID No: 1) (Maniatis et al., 1982, In Molecular Cloning: A laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.; Sanger et al., 1977, Proc.Natl.Acad.Sci. USA 74, 5463-5467). Comparison of the translated protein sequence with sequences held in the EMBL Database showed

surprisingly that it shared 88% homology with the sequence of the htrA product from E.coli (Fig.1. SEQ ID No: 1).

Example 2

Identification of htrA in S.typhimurium as a gene involved in the stress-response

E.Coli mutants harbouring lesions in the htrA gene are unable to grow at temperatures above 42°C. The S.typhimurium htrA mutant, 046, was tested for growth at elevated temperatures and was found to grow as well as the present strain C5. However, when tested for sensitivity to oxygen radicals, the mutant 046 showed decreased resistance as compared with the parent C5 strain clearly indicating that the gene is responsible (at least in part) for this aspect of the stress response (see Fig. 2).

Example 3

Comparison of attenuated Salmonella typhimurium strain 046 with virulent parent strain Salmonella typhimurium C5.

The attenuated strains were constructed using TnphoA transposon mutagenesis as described previously (Miller et al., 1989, Infect. Immun. 57, 2758-2763).

After oral administration the mutant strain 046 had a $\text{Log}_{10} \text{LD}_{50}$ of greater than 9 cells as compared to the parental strain, C5, which has a $\text{Log}_{10} \text{LD}_{50}$ of 6.38 cells. (All LD_{50} were calculated after 28 days). Thus 046 is highly attenuated. After i.v. administration 046 had an i.v. $\text{Log}_{10} \text{LD}_{50}$ of 5.13 cells compared to less than 10 cells for C5 and we again conclude that 046 is highly attenuated compared to C5.

Example 4

Protection of mice after oral challenge.

Mice were immunised with 046 and challenged 28 days later with the virulent parental strain C5. Mice vaccinated with using 10^{10} cells of 046 showed excellent protection against challenge with C5. eleven weeks after vaccination. The $\text{Log}_{10} \text{LD}_{50}$ in immunised animals was 9.64 cells compared with 6.6 cells for unimmunised controls. Thus, mice vaccinated orally with a single dose of 046 were well protected against virulent C5 challenge.

Example 5Construction of a defined S.typhimurium SL1344 htrA mutant

Sequence data facilitated the identification of suitable restriction endonuclease sites that could be used to introduce a deletion into the htrA gene. A 1.2Kb deletion was introduced by digesting with EcoRV and religating. A drug resistant marker was also introduced into the gene (Kanamycin cassette, Pharmacia) by standard techniques to enable selection for the presence of the deleted gene. The plasmid harbouring the deleted htrA gene was introduced into a polA strain S.typhimurium (BRD207) in which the plasmid cannot replicate. The only way that kanamycin resistance can be maintained in the host is if there has been a recombination event between the S.typhimurium sequences on the vector and the homologous regions on the chromosome. Loss of ampicillin resistance while maintaining kanamycin resistance indicates a second homologous recombination event resulting in the replacement of the intact htrA gene with the deleted one. Colonies resistant to kanamycin were isolated and checked for ampicillin resistance. One colony that was kanamycin resistant and ampicillin sensitive was selected for further study and was designated BRD698 (deposited at PHLS, NCTC, 61 Colindale Avenue, London NW9 5HT under

Accession No..... on in accordance with the terms of the Budapest Treaty).

A P22 lysate was prepared on this strain by standard techniques (Dougan *et al*, J.Infect.Dis. 158, 1329-1335, 1988) and used to infect SL1344. Kanamycin resistant colonies were isolated and checked for the presence of the deletion by Southern hybridisation. One strain, designated BRD726 (deposited at PHLS under Accession No. on in accordance with the terms of the Budapest Treaty) was selected for further study.

Example 6

Construction of an S.typhimurium SL1344 aroA htrA double mutant

The P22 lysate prepared on BRD698 was used to introduce the htrA deletion into an S.typhimurium SL1344 strain already harbouring a deletion in aroA. The method for introducing an aroA deletion has already been described by Dougan *et al*, J.Infect.Dis. 158, 1329-1335, 1988. One strain that was found to have deletions in both aroA and htrA was selected for further study and was designated BRD807, (deposited at PHLS under Accession No..... on in accordance with the terms of the Budapest Treaty).

Example 7

Comparison of the attenuation of SL1344 htrA (BRD726) and SL1344 htrA and aroA (BRD807) with the virulent parent strain SL1344

After oral administration BRD726 and BRD807 had Log_{10} LD_{50} s of >10.0 cells compared to the virulent parent strain which has a Log_{10} LD_{50} of 6.8 cells*. Both strains were therefore highly attenuated compared to the virulent parent strain SL1344.

*all LD_{50} s were calculated after 28 days.

Example 8Assessment of oral vaccine potential of BRD726 and BRD807

BALB/c mice were orally immunised with approximately 10^{10} cells of BRD726 and BRD807 as previously described (Dougan *et al.*, J.Infect.Dis. 158, 1329-1335, 1988) and challenged 4 and 10 weeks later with the virulent parent strain SL1344. LD₅₀s were calculated by the method of Reed and Muench (Am.J.Hyg. 27, 493-497, 1934). All determinations were carried out at least twice. Mice vaccinated with BRD726 and BRD807 showed excellent protection against challenge with SL1344 at 4 weeks, the log₁₀ LD₅₀s being >10.0 and 9.7 cells respectively. This compares with log 6.1 cells for unimmunised controls. At 10 weeks log₁₀ LD₅₀s for BRD726 and BRD807 were 9.11 and 8.11 cells compared to 6.5 for SL1344. Thus the mice immunised with BRD726 had excellent long term immunity to virulent SL1344 challenge. This compares favourably with protection elicited by double aro mutants of SL1344 (Dougan *et al.*, J.Infect.Dis. 158, 1329-1335, 1988). The long term protection afforded by vaccination with BRD807 is 46-fold better than unimmunised controls. Thus both BRD726 and BRD807 make good vaccine strains for BALB/c mice.

Example 9In vivo kinetics of BRD726 and BRD807 in BALB/c mice

The ability of BRD726 and BRD807 to grow in vivo after intravenous administration was assessed. Mice were infected with approximately 10^5 organisms. Numbers of bacteria in livers and spleens were enumerated at different times during the infection up to 21 days. The results obtained are shown in Fig3. Neither BRD726 or BRD807 underwent an initial period of replication in murine tissues. The strains are cleared slowly from the organs and by day 21 BRD807 has almost cleared from the murine tissues while BRD726 is still persisting at low levels.

Example 10**Formulation**

An attenuated microorganism of the present invention is preferably presented in an oral tablet form.

<u>INGREDIENT</u>	<u>MG/TABLET</u>
<u>Core tablets</u>	
1. Freeze-dried excipient carrier containing 10 ⁹ 10 ¹⁰ attenuated bacteria.	70.0
2. Silica dioxide (Aerosil 200)	0.5
3. Dipac (97% sucrose)	235.0
4. Cross-linked Povidone (Kollidon CL)	7.0
5. Microcrystalline Cellulose (Avicel pH102)	35.0
6. Magnesium Stearate	2.5
<u>Coating</u>	
7. Opadry Enteric, OY-P-7156 (Polyvinyl acetate phthalate + Diethylphthate)	35.0
	<hr/> 385.0 <hr/>

A carrier containing 5% sucrose, 1% sodium glutamate and 1% bacto casitone in an aqueous solvent is prepared. The organisms are suspended in this carrier and then subjected to freeze-drying.

The freeze-dried material is blended with Aerosil 200 and the blended mixture is sifted through a screen. The sifted powder is mixed with Dipac, Kolidan CL, Aricel pH102 and Magnesium Stearate in a blender. This blend is compressed into tablets for subsequent enteric coatings.

The skilled man will appreciate that many of the ingredients in this formulation could be replaced by functionally equivalent pharmaceutically acceptable excipients.

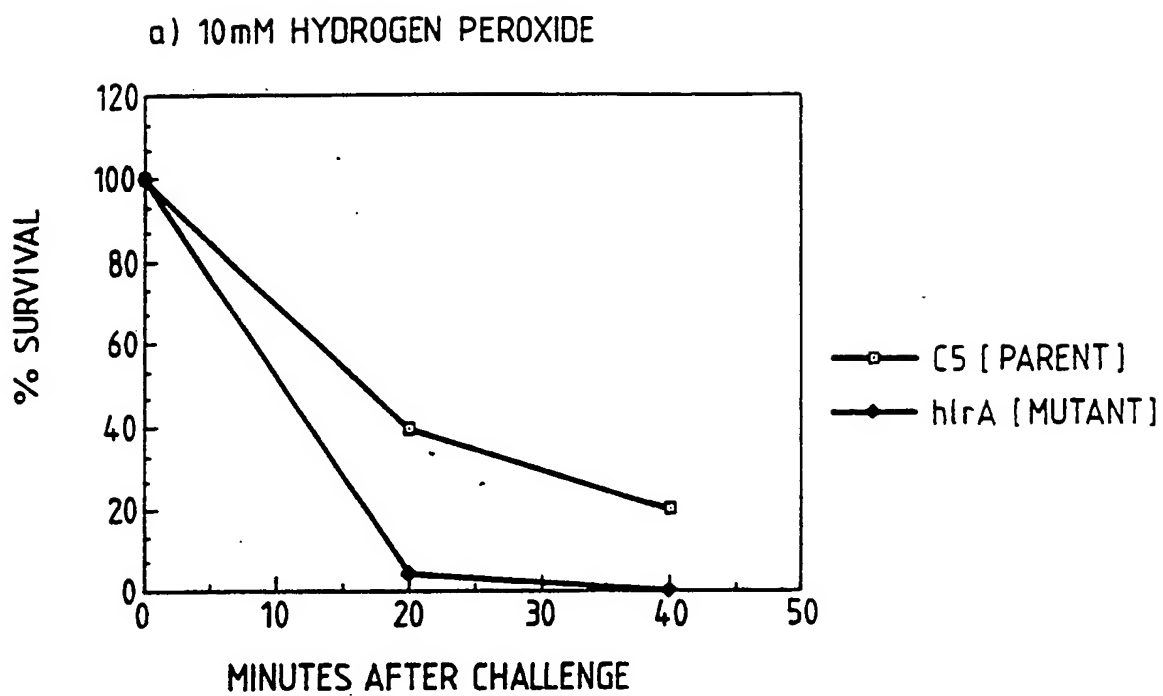
Claims

1. Attenuated microorganism for use in immunoprophylaxis in which the attenuation is brought about by the presence of a mutation in the DNA sequence of the microorganism which encodes, or which regulates the expression of DNA encoding a protein that is produced in response to environmental stress, the microorganism optionally being capable of expressing DNA encoding a heterologous antigen.
2. Attenuated microorganism as claimed in claim 1, wherein the protein is selected from a heat shock protein, a nutrient deprivation protein, a toxic stress protein and a metabolic distress protein.
3. Attenuated microorganism as claimed in claim 2, wherein the protein is a heat shock protein.
4. Attenuated microorganism as claimed in claim 3, wherein the protein is encoded by the htrA gene.
5. Attenuated microorganism as claimed in claim 1, wherein the mutation is a deletion or insertion mutation.
6. Attenuated microorganism as claimed in claim 1 wherein the microorganism is a bacterium.
7. Attenuated microorganism as claimed in claim 6, wherein the bacterium is selected from the genera Salmonella, Bordetella, Vibrio, Haemophilus and Escherichia.
8. Attenuated microorganism as claimed in claim 7, wherein the Salmonella bacterium is selected from Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis and Salmonella choleraesuis.

9. Attenuated microorganism as claimed in claim 1, in which the attenuation is also brought about by a mutation in a second DNA sequence.
10. Attenuated microorganism as claimed in claim 9, wherein the mutation in a second DNA sequence is in a DNA sequence involved in the aromatic amino acid biosynthetic pathway.
11. Attenuated microorganism as claimed in claim 10, wherein the DNA sequence involved in the aromatic amino acid biosynthetic pathway is selected from aroC, aroA and aroD.
12. Attenuated microorganism as claimed in claim 1 being capable of expressing DNA encoding a heterologous antigen.
13. A vaccine comprising an effective amount of attenuated microorganism, as claimed in any of claims 1-12, and a pharmaceutically acceptable carrier therefor.
14. A vaccine as claimed in claim 13 adapted for oral administration.
15. A method of prophylactic treatment of a host with an infection caused by a microorganism, which comprises administering to said host an effective dose of a vaccine as claimed in claim 13.
16. A process for the production of a microorganism as claimed in claim 1, which comprises introduction of a mutation in the DNA sequence of the microorganism which encodes or which regulates the expression of DNA encoding, a protein that is produced in response to environmental stress by either:
 - a) transposon mutagenesis, or
 - b) transforming the microorganism with a vector incorporating the DNA sequence encoding, or regulating the expression of a DNA sequence encoding a protein that is produced in response

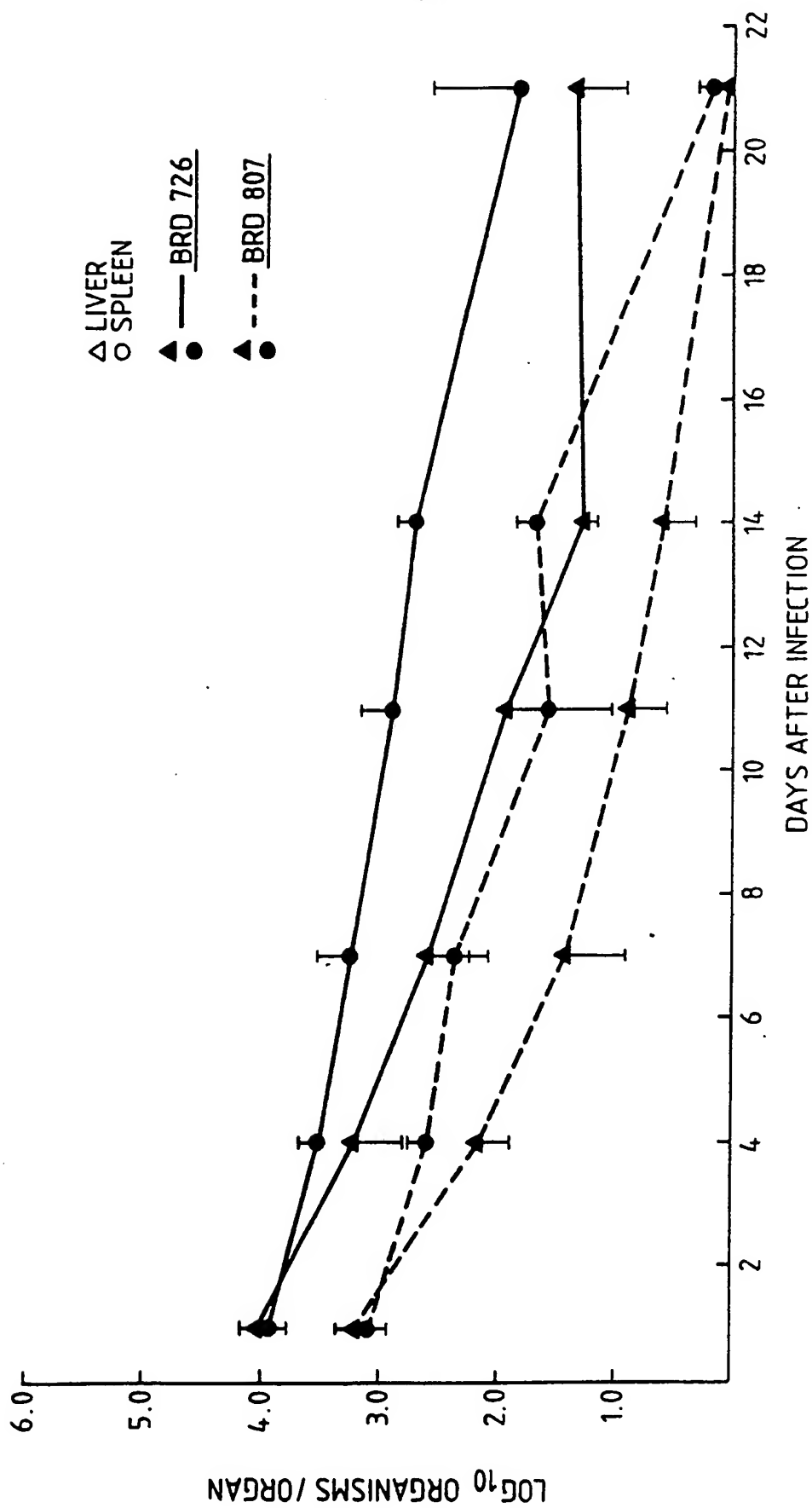
to environmental stress and which contains a non-reverting mutation; and

- c) screening to select the desired microorganisms.

Fig.2.

4/4

Fig. 3.



INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 91/00484

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC C 12 N 1/20, C 12 N 1/21, A 61 K 39/112 // A 61 K 39/10, IPC ⁵ : A 61 K 39/102, A 61 K 39/106, A 61 K 39/108														
II. FIELDS SEARCHED <div style="text-align: center; font-size: small;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; border: none;">Classification System</td> <td style="border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; padding: 5px;">IPC⁵</td> <td style="border: none; padding: 5px;">C 12 N, A 61 K</td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁵	C 12 N, A 61 K								
Classification System	Classification Symbols													
IPC ⁵	C 12 N, A 61 K													
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border: none;"> <tr> <th style="width: 10%; border: none; font-size: x-small;">Category ¹⁰</th> <th style="width: 70%; border: none; font-size: x-small;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border: none; font-size: x-small;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="border: none; text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="border: none; padding: 5px;">Infection and Immunity, volume 57, no. 9, September 1989, American Society for Microbiology, (US), I. Miller et al.: "Isolation of orally attenuated <u>Salmonella typhimurium</u> following TnpHoA mutagenesis", pages 2758-2763 see page 2760, column 1, line 19 - column 2, line 11; page 2761, column 2, last paragraph</td> <td style="border: none; text-align: center; vertical-align: top; padding: 5px;">1-8,12-14,16</td> </tr> <tr> <td style="border: none; text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="border: none; padding: 5px;">cited in the application ---</td> <td style="border: none; text-align: center; vertical-align: top; padding: 5px;">9-11</td> </tr> <tr> <td style="border: none; text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="border: none; padding: 5px;">Journal of Bacteriology, volume 171, no. 3, March 1989, American Society for Microbiology, (US), B. Lipinska et al.: "Identification, characterization, and mapping of the <u>Escherichia coli</u> htrA gene, whose product is essential for bacterial growth only at elevated temperatures", pages 1574-1584 see the whole document cited in the application --- ./.</td> <td style="border: none; text-align: center; vertical-align: top; padding: 5px;">1-7,12-14,16</td> </tr> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	Infection and Immunity, volume 57, no. 9, September 1989, American Society for Microbiology, (US), I. Miller et al.: "Isolation of orally attenuated <u>Salmonella typhimurium</u> following TnpHoA mutagenesis", pages 2758-2763 see page 2760, column 1, line 19 - column 2, line 11; page 2761, column 2, last paragraph	1-8,12-14,16	Y	cited in the application ---	9-11	X	Journal of Bacteriology, volume 171, no. 3, March 1989, American Society for Microbiology, (US), B. Lipinska et al.: "Identification, characterization, and mapping of the <u>Escherichia coli</u> htrA gene, whose product is essential for bacterial growth only at elevated temperatures", pages 1574-1584 see the whole document cited in the application --- ./.	1-7,12-14,16
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³												
X	Infection and Immunity, volume 57, no. 9, September 1989, American Society for Microbiology, (US), I. Miller et al.: "Isolation of orally attenuated <u>Salmonella typhimurium</u> following TnpHoA mutagenesis", pages 2758-2763 see page 2760, column 1, line 19 - column 2, line 11; page 2761, column 2, last paragraph	1-8,12-14,16												
Y	cited in the application ---	9-11												
X	Journal of Bacteriology, volume 171, no. 3, March 1989, American Society for Microbiology, (US), B. Lipinska et al.: "Identification, characterization, and mapping of the <u>Escherichia coli</u> htrA gene, whose product is essential for bacterial growth only at elevated temperatures", pages 1574-1584 see the whole document cited in the application --- ./.	1-7,12-14,16												
<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; padding: 5px;"> Date of the Actual Completion of the International Search 1st July 1991 </td> <td style="width: 50%; border: none; padding: 5px;"> Date of Mailing of this International Search Report 22.08.91 </td> </tr> <tr> <td style="border: none; padding: 5px;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="border: none; padding: 5px;"> Signature of Authorized Officer Danielle van der Haas </td> </tr> </table>			Date of the Actual Completion of the International Search 1st July 1991	Date of Mailing of this International Search Report 22.08.91	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer Danielle van der Haas								
Date of the Actual Completion of the International Search 1st July 1991	Date of Mailing of this International Search Report 22.08.91													
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer Danielle van der Haas													

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	WO, A, 88/05821 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 11 August 1988 see the whole document ---	1-7,12-14,16
X	Journal of Bacteriology, volume 171, no. 5, May 1989, American Society for Microbiology, (US), B. Bukau et al.: "Cellular defects caused by deletion of the <u>Escherichia coli</u> dnaK gene indicate roles for heat shock protein in normal metabolism", pages 2337-2346 see the whole document ---	1-3,4-7,12-14,16
X	Genes and Development, volume 2, 1988, N. Kusakawa et al.: "Heat shock protein GroE of <u>Escherichia coli</u> : key protective roles against thermal stress", pages 874-882 see page 875, column 1 ---	1-3,4-7,12-14,16
X	The EMBO Journal, volume 8, no. 11, 1989, IRL Press, (Oxford, GB), N. Kusakawa et al.: "Effects of mutations in heat-shock genes groES and groEL on protein export in <u>Escherichia coli</u> ", pages 3517-3521 see the whole document ---	1-3,4-7,12-14,16
X	Proc. Natl. Acad. Sci., volume 86, 1989, (US) E.A. Groisman et al.: " <u>Salmonella typhimurium</u> phoP virulence gene is a transcriptional regulator", pages 7077-7081 see the whole document ---	1,2,5-8,12-14,16
X	Journal of Bacteriology, volume 172, no. 2, February 1990, American Society for Microbiology, (US), J.W. Foster et al.: "Adaptive acidification tolerance response of <u>Salmonella typhimurium</u> ", pages 771-778 see table I ---	1,2,5-8,12-14,16
Y	Vaccine, volume 7, no. 6, December 1989, Butterworth & Co., (London, GB), S.N. Chatfield et al.: "Live <u>Salmonella</u> as vaccines and carriers of foreign antigenic determinants", pages 495-498 see page 495, column 2, line 20 - page 497, column 1, line 8 ---	9-11
	./.	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

T Biological Abstracts, volume 91,
K. Johnson et al.: "The role of a
stress-response protein in Salmonella
typhimurium virulence", see abstract
119974
& Mol. Microbiol. 5(2), 1991, 401-408

1

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 15 because they relate to subject matter not required to be searched by this Authority, namely:

Pls. see rule 39.1(iv) - PCT:
Methods for treatment of the human or animal body by surgery
or therapy, as well as diagnostic methods.

2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

SA 45885

EPO FORM 10479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82